Journal of the American Association for Laboratory Animal Science
Copyright 2009
by the American Association for Laboratory Animal Science

Vol 48, No 6
November 2009
Pages 718–726

Beyond-Use Dating of Extemporaneously Compounded Ketamine, Acepromazine, and Xylazine: Safety, Stability, and Efficacy over Time

Brett J Taylor,* Steven A Orr, Jennifer L Chapman, and Diana E Fisher

Ketamine–acepromazine–xylazine (KAX) has long been a popular combination of injectable anesthetics for use in laboratory rodents. These drugs are compounded extemporaneously at research facilities because a commercial mixture is not available. This study was designed to determine an appropriate period of use for this mixture by examining its safety, stability, and efficacy at 30-d intervals over an aging period of 270 d. For as long as 270 d after compounding, most of the data collected (chemical stability, sterility, pH, particulate formation, times to loss of righting reflex in injected mice and rats, and histopathology from these animals) supported the finding that the component drugs do not change or degrade. However, mice and rats did show significant differences in anesthetic responses after injection with KAX mixtures of different ages. In light of these findings, we suggest that KAX remains safe, stable, and efficacious for at least 180 d after mixing, and that 180 d constitutes an appropriate period of use for this drug combination when stored in a dark, room-temperature environment.

Abbreviations: KAX, ketamine–acepromazine–xylazine; USP, United States Pharmacopeia.

The combination of ketamine, acepromazine, and xylazine (KAX) is widely regarded as the agent of first choice for injectable rodent anesthesia and has been used in both rabbits and rodents in numerous published studies spanning many years of research.[2-4,6,7,11,13,15-17,22-24,26,27,30,37,40-45] Advantages of this particular anesthetic combination include its reliable dose-dependent loss of consciousness and muscle relaxation, its preservation of spontaneous ventilation, its wide margin of safety, and the physical compatibility of its component drugs.

A commercially produced combination of these drugs is not currently available from any pharmaceutical company in the United States. Research facilities that use KAX in rodents mix the drugs according to professional experience or institutional standard operating procedure, rather than according to specific instructions by a commercial manufacturer or definitive literature on the topic. In the field of pharmacology, this is known as extemporaneous compounding—the mixing of component drugs to produce a suitable medication for a specific purpose when no commercial forms are available.[20] Extemporaneously compounded drugs are assigned a beyond-use date, rather than an expiration date. The beyond-use date is defined as the end of that period of time over which an extemporaneously compounded drug has been shown to remain free of interactions between its individual component drugs and free of changes to its sterility and physical condition. The beyond-use date of a compounded mixture never encompasses a longer period of time than the expiration date of any of the component drugs.[9,39]

Through informal conversations with people from several other research institutes in the United States, we learned of a wide variety of beyond-use dating periods for KAX, ranging in length from 7 to 90 d. The variety in these time periods likely is due to the lack of published findings concerning the decomposition and shelf life of this solution. For approximately a decade, our institute has been assigning a beyond-use dating period of 90 d for this mixture (as long as the component drugs themselves do not expire before this time). At the time this study was submitted for publication, there has been no reported increase in the rate of adverse anesthetic effects and no reported loss of clinical efficacy observed with KAX as it ages to approach this 90-d beyond-use date.

The United States Pharmacopeia (USP) states that the only truly valid method of predicting a beyond-use date for a compounded product is to perform an experimental stability study.[39] Stability is defined as "the extent to which a product retains, within specified limits, and throughout its period of storage and use, the same properties and characteristics that it possessed at the time of its manufacture."[39] The USP recognizes 5 key aspects of this definition that also encompass safety and efficacy. That is, each active ingredient retains its chemical integrity and labeled potency, within the limits specified by its USP monograph (chemical stability); its original physical properties, including appearance, palatability, uniformity, dissolution, and suspendability (physical stability); its sterility or resistance to microbial growth, according to the specified requirements (microbiologic stability); and its therapeutic (in this case, anesthetic) effect (therapeutic stability); and it exhibits no significant increase in toxicity (toxicologic stability).[39]

From previous experience with this drug solution at up to 90 d of age, coupled with a high-pressure liquid chromatography pilot study, we hypothesized that KAX has an appropriate beyond-use dating period of at least 180 d. This study was designed in 5 distinct sections, to examine these aspects of safety, stability, and efficacy in KAX over time.

Received: 21 May 2009. Revision requested: 17 Jun 2009. Accepted: 19 Jun 2009.
The United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Maryland.
**Corresponding author. Email: brett.taylor@us.army.mil*

Materials and Methods

Animals and housing. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory* Animals.[18] The facility where this research was conducted is fully AAALAC-accredited. The Institutional Animal Care and Use Committee of the United States Army Medical Research Institute of Infectious Disease approved all procedures.

BALB/c mice were obtained from the National Cancer Institute (Fort Detrick, MD), and Brown Norway rats were obtained from Charles River (Kingston, NY). Animals of both species were 4-wk-old, all were male, and all were acclimated for 7 d before use in experiments. On arrival, the animals were free of viral, bacterial, and parasitic diseases, and their health was monitored over the course of these experiments under the institutional animal health surveillance program. Mice were housed in filter-topped polysulfone rodent cages, and rats were housed in a HEPA-filtered ventilated rack system, with 55 to 60 air changes per hour. Tap water was provided through sipper bottles to the mouse pans and through the automatic watering system to the ventilated racks containing the rats. All animals received a standard pelleted rodent diet (7022, Harlan Laboratories, Indianapolis, IN) and were housed on absorbent bedding material (Alpha-Dri, Shepherd Specialty Papers, Portage, MI). The light cycle was maintained at 12 h light and 12 h dark, the room temperature was kept at 23.3 ± 2.2 °C (72 ± 4 °F), and the room humidity was kept within the range of 30% to 70%.

Preparation and aging conditions of KAX solution. The components for each KAX solution were mixed from the original commercially manufactured drug bottles according to the established standard operating procedure at our institute. In accordance with this procedure, KAX is prepared at 2 concentrations, a full-strength version for use in rats and hamsters (called 'rat KAX'), and a 1:10 saline-diluted version for use in mice (called 'mouse KAX').

Rat KAX. To prepare this solution, 5.0 mL ketamine (100 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA) was drawn into a 5-mL syringe by using a 22-gauge needle and then injected through the rubber top into a clear, glass, additive-free 10-mL blood collection tube (Monoject 'red-top tube', Tyco Healthcare, Mansfield, MA). Then 0.5 mL acepromazine (10 mg/mL; Phoenix Pharmaceuticals, St Joseph, MO) was drawn into a 1-mL syringe by using a 22-gauge needle and injected into the same glass tube. Finally 2.75 mL xylazine (20 mg/mL; Lloyd Laboratories, Shenandoah, IA) was drawn into a 3-mL syringe by using a 22-gauge needle and injected into the same glass tube. This glass tube was labeled 'rat KAX' and inverted several times to ensure thorough mixture of the contents. At this point, the tube contained a calculated total volume of 8.25 mL.

Mouse KAX. To prepare this solution, 1.0 mL rat KAX was withdrawn from its tube into a 1-mL syringe with a 22-gauge needle and transferred into a new clear, additive-free, glass blood collection tube from the same manufacturer (Tyco Healthcare) and of the same production lot. Then 9.0 mL 0.9% sterile saline (Baxter, Deerfield, IL) was drawn into a 10-mL syringe by using an 18-gauge needle and injected into this same new glass tube. This tube was labeled 'mouse KAX' and inverted several times to ensure thorough mixture of the contents. This tube contained a total volume of 10 mL; the final calculated volume of the rat KAX tube (after the removal of 1.0 mL) was 7.25 mL.

The rubber stoppers on each bottle were wiped once with an alcohol swab before needle puncture. Rubber stoppers on KAX tubes also were wiped only once, although they were punctured with multiple needles during the process of compounding as described above. In keeping with institutional standard operating procedures, no additional efforts were made to achieve pharmaceutical-grade aseptic technique (for example, laminar flow hoods, clean rooms certified by the International Organization for Standardization, sterile protective garments).

By calculation, rat KAX mixed by these procedures contained ketamine at 60.6 mg/mL, acepromazine at 0.6 mg/mL, and xylazine at 6.67 mg/mL, whereas the concentrations of these same drugs in mouse KAX were diluted 1:10 to 6.06, 0.06, and 0.667 mg/mL, respectively. The final dose per kilogram of body weight was the same in both species (60.6, 0.6, and 6.67 mg/kg, respectively), but the difference in concentration meant that mice received a 10-fold larger injection volume per kilogram of body weight than did the rats. This practice is convenient for more accurate dosing in these smaller animals.

Because these tubes contained ketamine, a Schedule III drug, they were stored inside a locked safe within the pharmacy at all times. A digital temperature and humidity recorder (Traceable, Control Company, Friendswood, TX) was placed adjacent to these tubes on the same shelf, and the minimum and maximum temperatures and humidity levels within the safe were monitored at 30-d intervals throughout the course of the study. The safe was not internally illuminated, therefore the only light source to which these KAX tubes were exposed was the fluorescent room lighting and then only during those brief periods when the safe door was opened for the routine business of the pharmacy.

Over a 270-d aging period, 2 KAX tubes (a 7.25-mL tube of rat KAX and a 10-mL tube of mouse KAX) were prepared at 30-d intervals, resulting in a total of 20 tubes of KAX: 1 for each concentration (rat and mouse) at each of 10 different ages (270, 240, 210, 180, 150, 120, 90, 60, 30, and 0 d) at the end of this aging process, The stability experiments described in the sections below were conducted on day 270, scheduled to coincide with the end of this timed aging process.

Chemical stability. The 20 test KAX solutions were analyzed by high-pressure liquid chromatography (model 1100, Agilent, Santa Clara, CA) with a diode array detector at a wavelength of 254 nm. The analyses were run over a reverse-phase C18 column stationary phase. The 2-part mobile phase consisted of (a) 25 mM phosphate buffer with 1% triethyl amine at pH 3.0 and (b) acetonitrile. The procedure was conducted at a linear gradient of 20% to 50% acetonitrile over 15 min.

In addition to ketamine, acepromazine, and xylazine, the KAX absorbance chromatogram demonstrated another peak at elution time 7.8 min (Figure 1). This compound was methylparaben, an antimicrobial preservative present in xylazine manufactured by Lloyd Laboratories. Because its presence is relevant to both the chemical and microbiologic stability of the compounded product, it was included in the analysis as well. Each of these 4 major KAX components exhibited highly reproducible elution times (relative standard deviations ≤ 1.5%).

A 6-point standard concentration curve of 50%, 70%, 85%, 100%, 125%, and 150% of the expected concentration was constructed for each drug component. Equations ($y = mx + b$) of chromatographic peak area versus drug concentration were determined to be both linear (with R^2 values greater than 0.99) and reproducible (with relative standard deviations of less than 0.5% for triplicate samples of each drug). These line equations then were used to calculate the concentrations of each component.

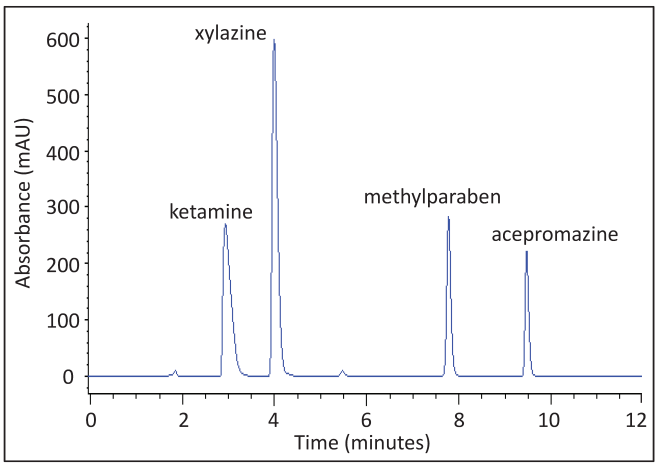

Figure 1. KAX Chromatogram generated from a sample of mouse KAX mixed immediately before analysis, demonstrating well-resolved peaks that are sufficiently separated from each other for identification and measurement.

Rat KAX was diluted 1:10 immediately before HPLC analysis so as to not overload the detector. This practice allowed the same concentration curves to be used for both mouse KAX and rat KAX. The concentrations of components measured in the rat KAX were multiplied by 10 after analysis, to calculate their concentrations in the original sample.

Therapeutic (anesthetic) stability. Of the 20 mixtures, 6 (the rat and mouse concentrations aged for 0, 180, and 270 d) were selected for evaluation of their efficacy and clinical safety in rats and mice. The 3 rat KAX solutions were tested in 3 groups of 10 rats, and the 3 mouse KAX solutions were tested in 3 groups of 10 mice. In addition, 3 mice and 6 rats were assigned to control groups and received injections containing 0.9% sterile saline at the same time and in the same volume per body weight as the KAX study animals. These animals served as controls only for the histopathologic evaluation of the animals after necropsy.

Each mouse and rat was weighed individually immediately before dosing. Two injections were given, 3 d apart, and their responses to the anesthetic were evaluated on both occasions. The first injection was given intramuscularly into the left caudal thigh, and the second injection (3 d later, from the same tube of KAX solution) was given intraperitoneally into the right side of the abdomen, with the animal restrained in the Trendelenberg position. The interval of 3 d between these injections was chosen in to simulate other recent research protocols at this institute that have used KAX anesthesia, and the use of the 2 different administration routes allowed for the comparison of effects between them. All injections were given by using 1-mL tuberculin syringes and 25-gauge, 5/8-in. needles.

The evaluation of anesthetic efficacy relied on 2 observations: time until loss of righting reflex (defined as the cessation of purposeful righting motions upon placement into dorsal or lateral recumbency) and a combined response score that was measured at 5-min intervals. The combined response score consisted of the sum of 3 individual response measurements: the corneal drop response, abdominal flinch response, and hindlimb withdrawal response. These individual response measurements were adapted from those in previous publications.[2,4,12,34]

Hindlimb withdrawal response was evaluated as the strength and speed of withdrawal response to a mosquito hemostat closed on the interdigital webbing of the animal's rear feet. Corneal drop response was evaluated as the strength and speed of blinking or flinching in response to a single droplet of 0.9% saline from a syringe onto the animal's cornea. Abdominal flinch response was evaluated as the strength and speed of flinch or avoidance effort in response to a #11 scalpel blade gently pushed into the skin over the animal's ventral abdomen, without penetrating the outer layer of skin.

Each of these individual responses was scored on a scale of 0 to 2 (where 0 indicated the absence of a response, 1 indicated an intermediate response in terms of time or magnitude, and 2 indicated a normal response such as that observed in an awake animal). To obtain the combined response score for an animal, the 3 individual scores for each time point were added together to produce a combined score with a possible range of 0 to 6. This testing was done at 5-min intervals until the animal regained its righting reflex.

Toxicologic stability. Pathology was used to evaluate potential KAX-induced morphologic changes in target organs. A necropsy was performed on all animals after euthanasia 4 d after the second anesthetic event; the following tissues were collected: skull (to include brain, eyes, nasal cavity, and sinuses); liver; spleen; kidneys; and skeletal muscle from the left rear leg (site of intramuscular injection). Formalin-fixed tissues for histologic examination were trimmed, processed, and embedded in paraffin according to established protocols.[28] Histology sections were cut at 5 to 6 µm, mounted on glass slides, and stained with hematoxylin and eosin. The pathologist reviewed the slides without knowledge of the specific experimental groups but was aware of the animals in the saline control groups.

Microbiologic stability. The sterility of each tube of KAX was assessed by drawing 0.5 mL of its contents into fluid thioglycollate medium, incubating it at 32.5 ± 2.5 °C for 9 d, and then evaluating for the presence of microbial growth. This test was performed for all 20 KAX samples.

Physical stability. The pH of each sample was measured with a Corning 440 pH-meter (Corning Incorporated, Corning, New York, NY), calibrated at pH 4.0 and pH 10.0. During this process, the tubes were compared subjectively for color changes.

The final experiment was to look for the formation of crystals, sedimentation products, or other particulate debris within the compounded drugs. All 20 KAX samples were centrifuged in their tubes at approximately 2400 × *g* for 5 min. The rubber tops were removed, and a sample of approximately 0.01 mL was aspirated from the bottom of the tube into a tuberculin syringe. This syringe was emptied onto a glass slide with coverslip and examined under a light microscope with a 40× objective.

Statistical Analysis. All data were checked for normality and statistical analyses were performed by using SAS version 9.1 (SAS Institute, Cary, IL). If data were normally distributed, 1-way ANOVA and *t* tests were used. If data were not normally distributed, Kruskal–Wallis or Mann–Whitney *U* tests were used.

Results

KAX solutions and test animals. The temperature and humidity within the pharmacy safe, as recorded by the monitor, remained within the ranges of 20 to 26 °C and 27% to 57%, respectively, over the entire duration of this study. This information represents the temperature and humidity exposure of the KAX samples as well, as they were stored in close proximity to this monitor at all times (except when quantities were being drawn up for testing purposes).

Immediately before the start of the study, 2 rats were removed from the group assigned to receive the 270-d-old KAX because of unthriftiness of unknown cause. Subsequent necropsy of these animals was able to determine no specific lesions. In addition, 1

mouse was removed from the group assigned to receive 0-d-old KAX due to an accidental wastage of that animal's test drug. All other study groups consisted of 10 animals each.

Chemical stability. Chromatograms were used to calculate concentrations for the ketamine, acepromazine, xylazine, and methylparaben in each KAX sample (Figure 2). The data in this figure include an additional time point of 360 d, which was not tested in any of the other sections of this study. This time point actually represented a repeat testing of the 270-d drug samples, performed 90 d later, and was added after we obtained the results from testing at earlier time points.

Regression analysis was performed to determine the association between age of solution and calculated concentration for each chemical component. This analysis revealed a slight but significant increase in ketamine concentration in mouse KAX with age (slope = 0.00187, $P = 0.0460$). There were no other significant differences over time in concentrations of the component drugs in either the mouse KAX or the rat KAX.

Therapeutic (anesthetic) stability. ***Loss of righting reflex.*** Figure 3 illustrates the time elapsed from KAX injection to the loss of righting reflex in each species and for each injection route. For both rats and mice, ANOVA was used to detect differences between groups defined by the age of KAX solution they received, for each of the 2 injection routes independently. In addition, post hoc Tukey tests were performed to make pairwise comparisons between groups within each sample. Although the difference in time to loss of righting reflex between KAX at 0 d and 180 d when injected intramuscularly in mice closely approached statistical significance ($P = 0.0500$), there were no significant overall or pairwise differences determined between loss of righting reflex times within either species, regardless of the age of the KAX or the route of injection.

Combined response scores. The mean combined response score was calculated for each group of study animals using data collected at 5-min time points (Figure 4). Normality assumptions required for parametric methods were not met for these combined response score data. Thus the nonparametric analog of ANOVA, the Kruskal–Wallis test, was used to assess overall group differences among the 3 groups at each time point. This analysis was performed only for the time points up to 55 min, because some animals in each group were recovering their righting reflexes at that point.

Toxicologic stability. Postmortem examination was performed on all animals. Gross lesions were not observed in either the experimental or control groups. Histopathologic findings were present only in the skeletal muscle of the left rear leg associated with intramuscular injection in both the experimental (KAX) and control (saline) groups. The lesions in the remaining tissues examined were considered background changes or incidental findings and were not interpreted to be associated with KAX administration (data not shown).

Histologic findings associated with skeletal muscle in experimental animals included myositis and fasciitis; myocyte degeneration, necrosis, and loss; myocyte regeneration; and hemorrhage. The character of the inflammation in the skeletal muscle of mice was granulomatous, with macrophages and multinucleated giants cells, often with accompanying mineralization (Figure 5). The nature of the inflammation in rats ranged from lymphoplasmacytic and histiocytic to neutrophilic, with no mineralization. In addition, rats frequently exhibited fibrosis and granulation tissue associated with skeletal muscle lesions;

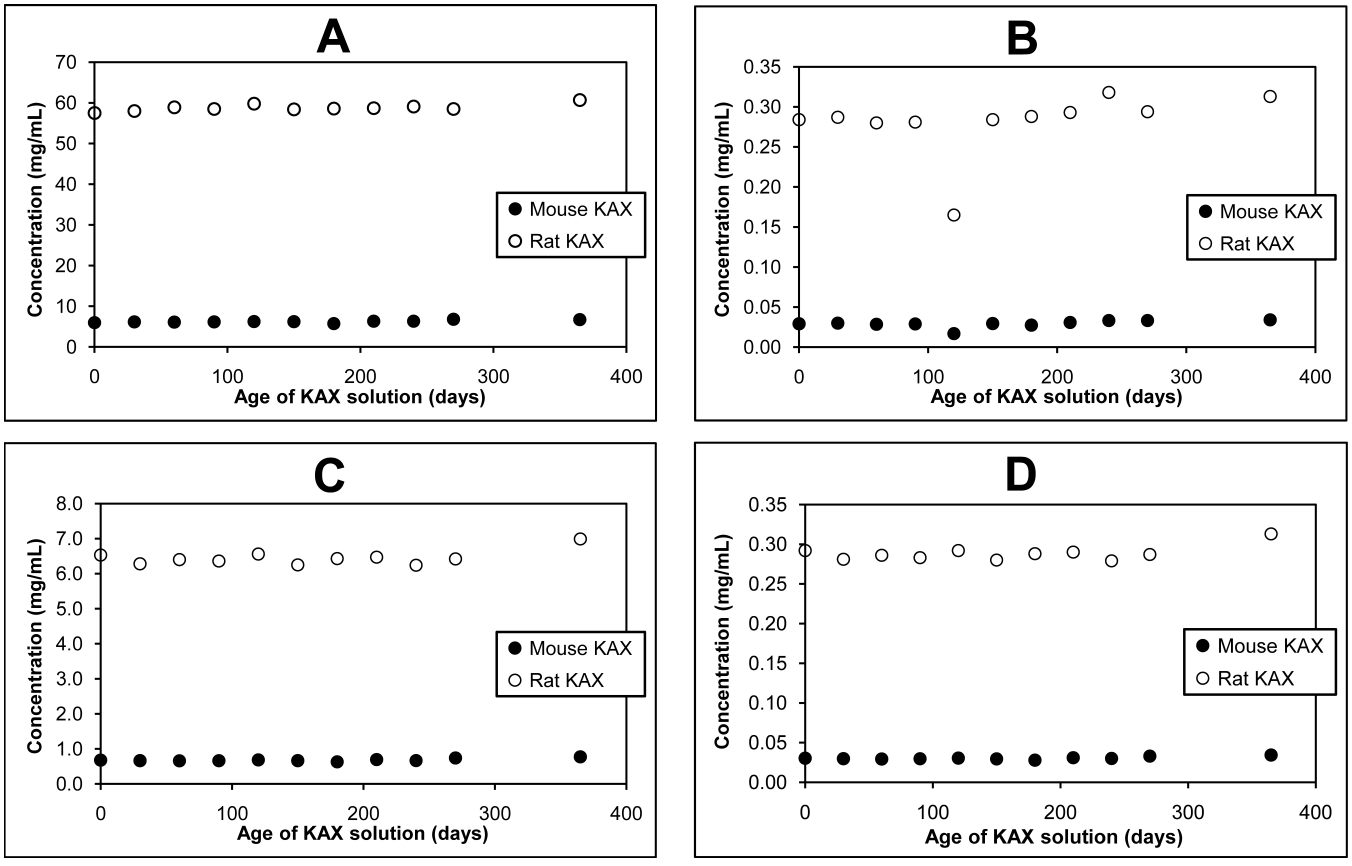

Figure 2. Analysis of concentrations of KAX components over time. (A) Ketamine. (B)Acepromazine. (C) Xylazine. (D) Methylparaben.

these changes were not found in mice. Minimal myositis and myocyte regeneration were sporadically present in the skeletal muscle associated with intramuscular injection of saline in control animals (data not shown).

Fisher exact tests were used to determine whether there was a statistically significant difference in the percentage of subjects presenting with a specified histopathologic finding among groups given KAX solutions aged 0, 180, or 270 d and control subjects. In mice, there were no statistically significant differences in the proportion of subjects exhibiting the aforementioned findings. In rats, there were statistically significant overall differences in the proportion of subjects with both subacute, chronic myositis and fibrosis between the control and study groups; however, there were no significant pairwise differences between any 2 study groups.

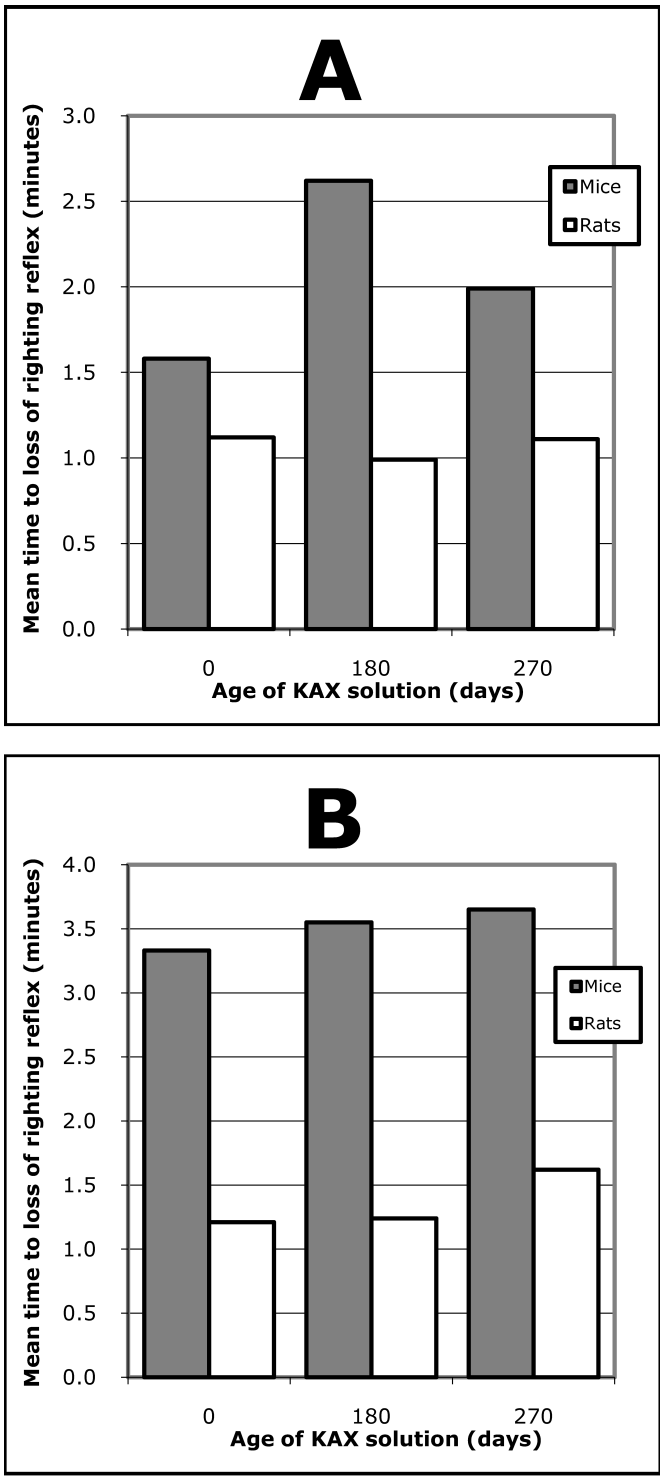

Figure 3. Time to loss of righting reflex in mice and rats after injection of KAX. (A) Intramuscular injection. (B) Intraperitoneal injection.

Microbiologic stability. None of the 10 rat KAX samples or the 10 mouse KAX samples displayed any bacterial or fungal growth after 9 d of incubation.

Physical stability. Regression analysis determined no statistically significant association between the age of solution and pH in KAX solutions at either concentration. The mean of the mouse KAX pH measurements was 5.07 (1 standard deviation, 0.0132 pH units), and the mean of the rat KAX pH measurements was 4.80 (1 standard deviation, 0.0126 pH units). No subjective color differences were noted between KAX compounds of different ages. After centrifugation of the glass tubes and subsequent examination by light microscopy, no crystals, sedimentation products, or other particulate debris was found in any of the 20 samples.

Discussion

This study consisted of distinct and separate experiments, each intended to evaluate the different aspects of safety, stability, and efficacy in extemporaneously compounded KAX solutions over time. This design constituted a thorough and well-rounded assessment for this purpose, and the results of the various individual experiments were consistent, with the exception of the combined response scores. No significant differences were noted among test groups in the chromatograms (to 360 d—longer than we originally had considered testing samples), times to loss of righting reflex, histopathologic findings, microbiologic stability, pH, or formation of particulates.

We hypothesized that the test animals would be likely to display more innate variability in response to the drugs than the drugs themselves would display in response to any subtle variations between compounding events. Therefore, in an effort to reduce the overall variability in the study as a whole, the drugs were prepared at consecutive 30-d intervals and the animals were used for testing all at once, rather than the converse. Compounds from all age groups were administered to the animals on the same day and at approximately the same time, to avoid previously documented differences in ketamine metabolism at different phases of the day.[31] Similarly, the use of all male animals avoided the documented difference in ketamine metabolism between the sexes.[5] Although this strategy may have reduced the variability observed between the animal groups receiving each compound, it did not eliminate it—as evidenced by numerous significant differences noted in the combined response scores (Figure 4). Fewer differences were detected among the groups in both species when the KAX was given intramuscularly as opposed to intraperitoneally. Aside from this point, these differences display no identifiable pattern, and no other coherent statements could be made addressing the relative efficacy of 1 compound over another at any given combination of time point, animal species, and injection route.

Anesthetic depth in laboratory rodents is difficult to objectively measure without specialized monitoring equipment. In the absence of access to such equipment, we designed a system to score the animals' responses to external stimuli. Two aspects of this design were intended to minimize subjectivity: first, a minimalistic scoring system was used, consisting of only 3 possible responses. With 0 defined as no response at all, and 2 defined as the degree of response observed in a fully awake animal, a response of 1 was the only scoring option for any intermediate response. Second, the stimulation used during

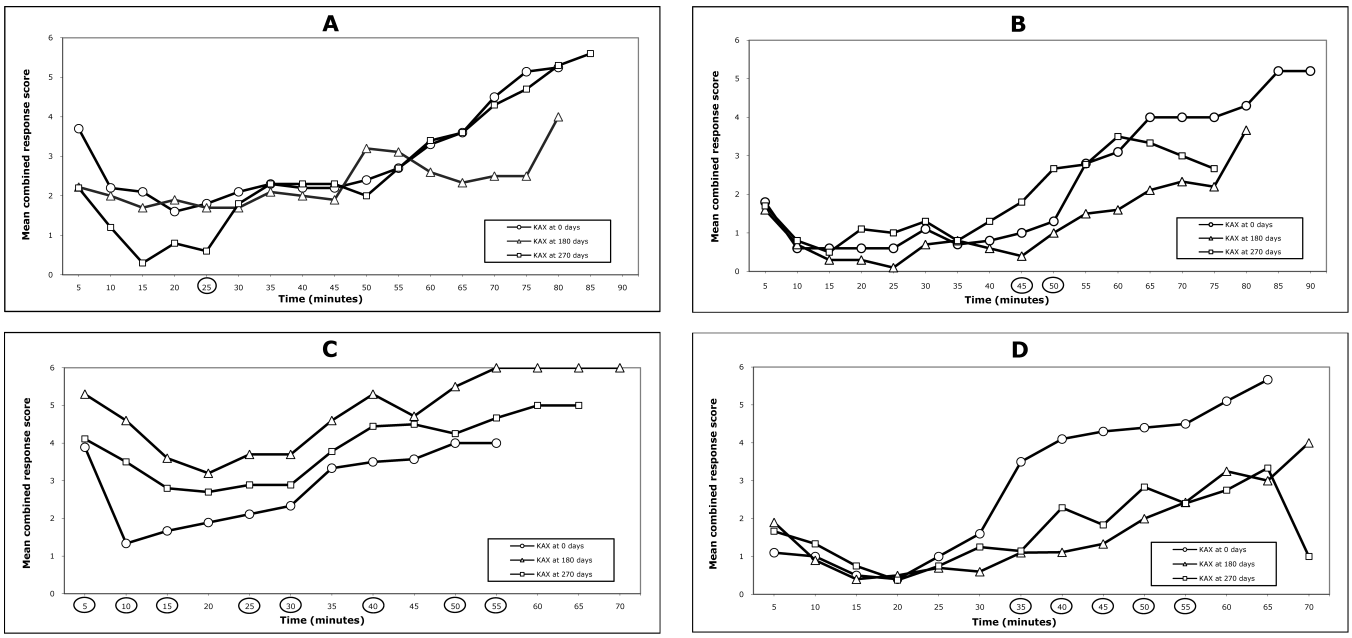

Figure 4. Combined response scores in (A, C) mice and (B, D) rats after (A, B) intramuscular and (C, D) intraperitoneal injection of KAX. Time points at which the difference among groups was statistically significant ($P < 0.05$) are indicated with a circle drawn beneath the *X* axis.

this experiment was as standardized as possible, with identical equipment used to produce the stimuli in each group and with training conducted for all scorers in advance. Ideally, a single person might have scored all animals across all groups to eliminate the possibility of observer bias on any 1 group; however, the number of animals involved and the need to perform all tests simultaneously precluded this possibility.

To evaluate potential KAX-induced morphologic changes, target organs were chosen based on previous reports of lesions induced by 1 or more of these drugs.[10,14,32,38] The only significant lesions related to experimental manipulation were observed histologically and were located in the skeletal muscle of the left hindlimb, caused by intramuscular injection of KAX solution in experimental animals. Lesions included skeletal muscle degeneration, necrosis, and loss; myocyte regeneration, myositis, or fasciitis; fibrosis; granulation tissue; and mineralization. Similar lesions have been reported to occur in rats and other laboratory animal species in association with intramuscular injection of ketamine alone or ketamine in combination with xylazine and acepromazine.[8,10,33,40] The low pH of ketamine has been implicated in the development of these localized skeletal muscle lesions.[8,10] Lesions in the control animals were minimal and consisted of myocyte regeneration or myositis, likely simply due to the introduction of the needle or saline solution. As in other studies, no lesions were present in the examined tissues in association with intraperitoneal injection of KAX, presumably due to the small volume administered and rapid absorption in the abdominal cavity.[33]

The inflammatory response in the muscle tissue of mice was primarily granulomatous, whereas the inflammation in rats was subacute to chronic or chronic–active with no granulomatous component. Although both species displayed features of chronic inflammation, the difference in the specific type of chronic inflammation suggests that each species may have a distinctive response to intramuscular injection. The granulomatous component in mice suggests a foreign body reaction to the deposited drug solution. These lesions also showed mineralization, which may represent dystrophic mineralization as a result of direct tissue injury and a subsequent foreign body inflammatory reaction. Alternatively some physical variable, such as the difference in volume of drug solution per kilogram of body weight (for more accurate dosing, a larger volume was administered to mice) may have caused the differences observed between the 2 species. Nonetheless, chronic inflammation is a predictable finding after intramuscular injection of KAX in multiple species.

Consistency in the results from each section of this study (with the exception of the anesthetic response scores) suggests that KAX remains physically and chemically unchanged for as long as 270 d. The anesthetic scores were the least objective group of data gathered in this study. These evaluations were included in anticipation of finding at least some degree of decomposition and were intended to reveal the clinical efficacy and toxicological effects of the compound at that particular level of decomposition. Because all experiments were conducted simultaneously, we could not predict the lack of decomposition of this compound in advance.

Results of the 360-d HPLC time point indicate that KAX may remain quite stable for even longer periods than 270 d. No additional testing was conducted to pursue this possibility, because there is no clear need at our institute to create this drug compound in such large quantities that it would benefit from a beyond-use date beyond 270 d. In addition, the longer the storage of the combined preparation, the greater is the potential for exposure to light and temperature outside the range experienced in the course of this study.

A clear potential for variability in the compounding of any liquid drug arises from the many volume measurements that must be performed. This situation is likely to have been responsible for the single outlier concentration at the 120-d time point on the acepromazine chromatogram. Each time the drugs in this study were mixed together into a tube or injected into test animals, the volume measurements were performed with a needle and syringe. Syringe sizes selected for these measurements were appropriate for the desired volumes, but there is some error inherent within these instruments. Recent publications have demonstrated a startling lack of precision in the use of 1-mL syringes, particularly at lower volumes such as those injected into animals during this study (0.1 to

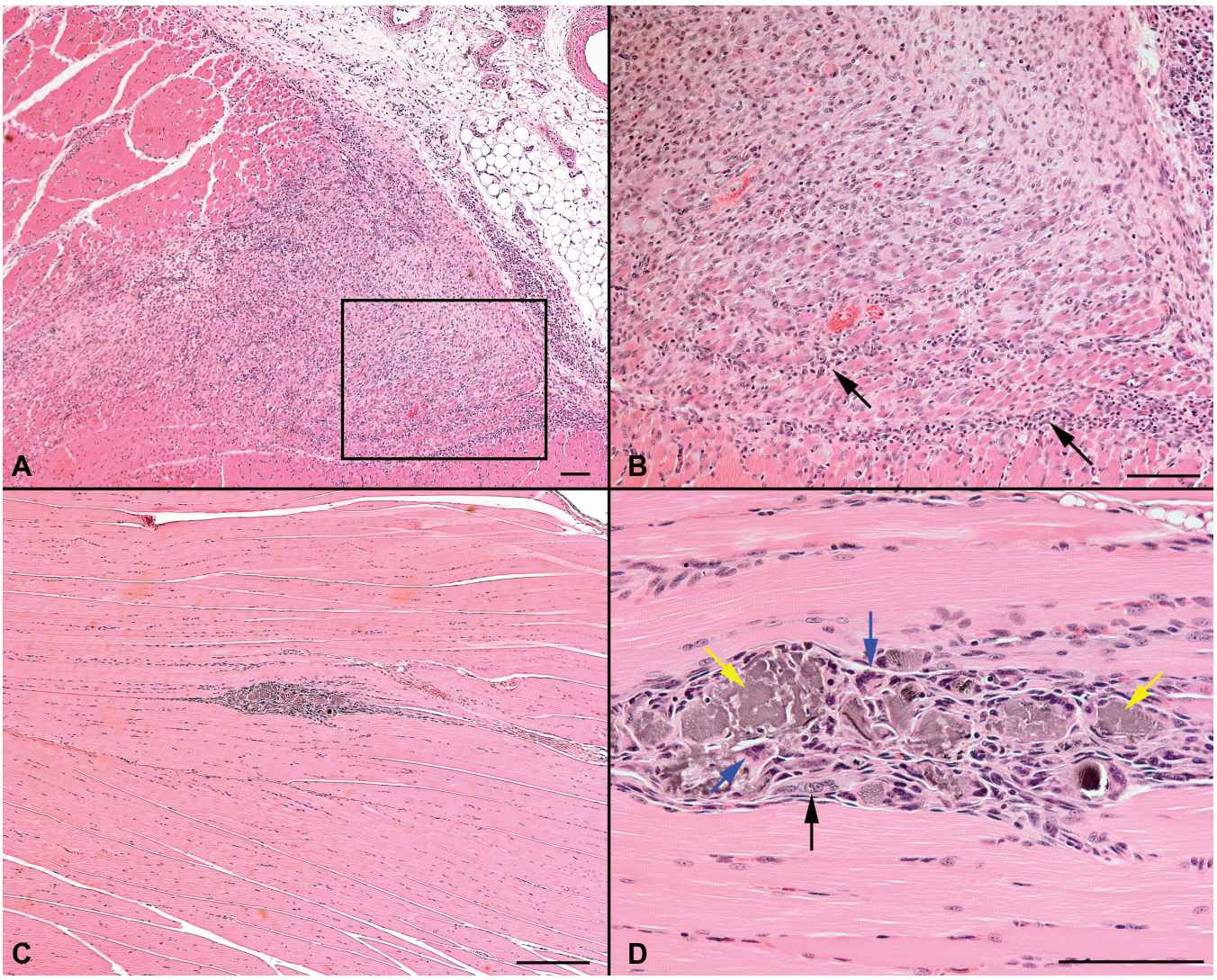

Figure 5. Skeletal muscle, left rear leg (site of intramuscular KAX injection). (A) Rat FR4. There is a focally extensive area of inflammation and fibrosis. Magnification, ×40. (B) Rat FR4. Depicted is the inset region of (A). Myocytes are degenerate to necrotic and are separated, surrounded, and replaced by fibroblasts and low numbers of lymphocytes and plasma cells (arrows). There is also myocyte regeneration. Magnification, ×100. (C) Mouse BM1. A focal area of inflammatory cells separates and replaces myocytes. Magnification, ×100. (D) Mouse BM1. Depicted is a higher magnification of (C). Granulomatous inflammation is composed of macrophages and multinucleated giant cells (blue arrows) with few lymphocytes and plasma cells. Note foci of amorphous granular material (mineralization, yellow arrows). There is also myocyte regeneration with central rowing of nuclei (black arrow). Magnification, ×200. Hematoxylin and eosin stain; bar, 20 µm.

0.2 mL).[21,25,29] Even for larger syringes, the International Organization for Standards has established allowable tolerances of approximately 5% error in volume measurements.[19] Every dose of mouse KAX was exposed to this potential for error on 5 occasions—3 times as each of the 3 component drugs were drawn up and mixed into a tube at the rat KAX concentration, a fourth time as 1.0 mL was drawn from this tube and diluted into sterile saline, and then finally a fifth time as the final dose was drawn up for administration. A quality control step, which could be as simple as weighing the tubes on a sensitive scale after they are mixed, could reduce this potential for error. Alternately, the use of a clinical refractometer has recently been described as an effective method of verifying the mixture of drug compounds.[36] These techniques could be used to test final mixtures of almost any liquid compounded medication, from anesthetics to antibiotics. As a final consideration, the use of volumetric pipettes could provide a much higher degree of accuracy and precision for the compounding process than that of syringes and needles.

Chapter 797 of the USP discusses several such quality control methods in depth, along with additional guidelines for the preparation and storage of compounded sterile products. Although this chapter is written with a clear emphasis toward the compounding of medications for human use, much of its guidance is also relevant to the practice of veterinary pharmacology. In fact, 2 national associations that represent the practice of veterinary pharmacy, the American Veterinary Medical Association and the Society of Veterinary Hospital Pharmacists, both reference USP standards in their position statements and standards of practice.[1,35] This recently developed chapter (first published in the USP in 2004) is an excellent source of guidance and advice concerning the use of compounded medications in research animals.

In summary, ketamine, acepromazine, and xylazine appear to be very stable when mixed together in solution and protected from light and excessive temperatures. The results of this study support the safety, stability, and efficacy of extemporaneously compounded KAX over a period of 270 d; however, as with

any substance, the potential for contamination or exposure to environmental extremes increases with the length of storage. With this caveat in mind, we recommend a conservative 180-d beyond-use dating period for this drug compound.

Acknowledgments

The authors declare no completing interests in this study. We thank Ms Gale Krietz, Mr Neil Davis, and Mr Jeff Brubaker for histology preparations; Dr J Edward Brown for the use of his laboratory facilities; the officers in the Veterinary Medicine Division for their advice and critical reviews; and the entire staff of USAMRIID animal technicians for their time and expertise.

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army.

References

1. **American Veterinary Medical Association.** [Internet]. Brochure on veterinary compounding. [Cited 30 Mar 2009]. Available at http://www.avma.org/issues/drugs/compounding/veterinary_compounding_brochure.asp.
2. **Arras M, Autenried P, Rettich A, Spaeni D, Rulicke T.** 2001. Optimization of intraperitoneal injection anesthesia in mice: drugs, dosages, adverse effects, and anesthesia depth. Comp Med **51:**443–456.
3. **Bui BV, Fortune B.** 2004. Ganglion cell contributions to the rat full-field electroretinogram. J Physiol **555:**153–173.
4. **Buitrago S, Martin TE, Tetens-Woodring J, Belicha-Villanueva A, Wilding GE.** 2008. Safety and efficacy of various combinations of injectable anesthetics in BALB/c mice. J Am Assoc Lab Anim Sci **47:**11–17.
5. **Cruz JI, Loste JM, Burzaco OH.** 1998. Observations on the use of medetomidine–ketamine and its reversal with atipamezole for chemical restraint in the mouse. Lab Anim **32:**18–22.
6. **Danias J, Kontiola AI, Filippopoulos T, Mittag T.** 2003. Method for the noninvasive measurement of intraocular pressure in mice. Invest Ophthalmol Vis Sci **44:**1138–1141.
7. **Davalos D, Lee JK, Smith WB, Brinkman B, Ellisman M, Zheng B, Akassoglou K.** 2008. Stable in vivo imaging of densely populated glia, axons, and blood vessels in the mouse spinal cord using 2-photon microscopy. J Neurosci Methods **169:**1–7.
8. **Davy CW, Trennery PN, Edmunds JG, Altman JFB, Eichler DA.** 1987. Local myotoxicity of ketamine hydrochloride in the marmoset. Lab Anim **21:**60–67.
9. **Food and Drug Administration.** [Internet]. Expiration dating of unit-dose repackaged drugs: compliance policy guide. Draft guidance, 2005. [Cited 30 Mar 2009]. Available at http://www.fda.gov/CDER/GUIDANCE/6169dft.pdf.
10. **Gaertner DJ, Boschert KR, Shoeb TR.** 1987. Muscle necrosis in Syrian hamsters resulting from intramuscular injections of ketamine and xylazine. Lab Anim Sci **37:**80–83.
11. **Gaertner DJ, Hallman TM, Hankenson FC, Batchelder MA.** 2008. Anesthesia and analgesia for laboratory rodents, p 239–282. In: Fish RE, Brown MJ, Danneman PJ, Karas AZ, editors. Anesthesia and analgesia in laboratory animals. San Diego (CA): Academic Press.
12. **Gardner DJ, Davis JA, Weina PJ, Theune B.** 1995. Comparison of tribromoethanol, ketamine/acetylpromazine, Telazol/xylazine, pentobarbital, and methoxyflurane anesthesia in HSD:ICR mice. Lab Anim Sci **45:**199–204.
13. **Hawk CT, Leary SL.** 1999. Formulary for laboratory animals, p 48. 2nd ed. Ames (IA): Iowa State University Press.
14. **Hayashi H, Dikkes P, Soriano SG.** 2002. Repeated administration of ketamine may lead to neuronal degeneration in the developing rat brain. Paediatr Anaesth **12:**770–774.
15. **Hobbs BA, Rolhall TG, Sprenkel TL, Anthony KL.** 1991. Comparison of several combinations for anesthesia in rabbits. Am J Vet Res **52:**669–674.
16. **Holcombe DJ, Lengefeld N, Gole GA, Barnett NL.** 2008. The effects of acute intraocular pressure elevation on rat retinal glutamate transport. Acta Opthalmol **86:**408–414.
17. **Huang CS, Boudinot FD, Feldman S.** 1995. Effects of gender, pregnancy, and anesthesia on the pharmacokinetics of zidovudine in rats. Pharm Res **12:**1647–1654.
18. **Institute for Laboratory Animal Research.** 1996. Guide for the care and use of laboratory animals. Washington (DC): National Academies Press.
19. **International Organization for Standardization.** 2006. Sterile hypodermic syringes for single use, part 1: syringes for manual use. ISO 7886-1:2006.
20. **Kairuz TE, Gargiulo D, Bunt C, Garg S.** 2007. Quality, safety, and efficacy in the 'off-label' use of medicines. Curr Drug Saf **2:**89–95.
21. **Keith K, Nicholson D, Rogers D.** 2004. Accuracy and precision of a low-dose insulin administration using syringes, pen injectors, and a pump. Clin Pediatr (Phila) **43:**69–74.
22. **Koulchitsky SV.** 1998. Are the capsaicin-sensitive structures of ventral medulla involved in the temperature response to endotoxin in rats? Neurosci Lett **244:**112–114.
23. **Lawson DM, Duke JL, Zammit TG, Collins HL, DiCarlo SE.** 2001. Recovery from carotid artery catheterization performed under various anesthetics in male Sprague–Dawley rats. Contemp Top Lab Anim Sci **40:**18–22.
24. **Lipman NS, Marini RP, Erdman SE.** 1990. A comparison of ketamine–xylazine and ketamine–xylazine–acepromazine anesthesia in the rabbit. Lab Anim Sci **40:**395–398.
25. **Lteif AN, Schwenk WF.** 1999. Accuracy of pen injectors versus insulin syringes in children with type 1 diabetes. Diabetes Care **22:**137–140.
26. **Mastronardi CA, Yu WH, McCann SM.** 2001. Lipopolysaccharide-induced tumor necrosis factor-alpha is controlled by the central nervous system. Neuroimmunomodulation **9:**148–156.
27. **Nagaraju M, Saleh M, Porciatti V.** 2007. IOP-dependent retinal ganglion cell dysfunction in glaucomatous DBA/2J mice. Invest Ophthalmol Vis Sci **48:**4573–4579.
28. **Prophet EB, Mills B, Arrington JB, Sobin LH; Armed Forces Institute of Pathology.** 1992. Laboratory methods for histotechnology, p25–29. Washington (DC): American Registry of Pathology.
29. **Raju JR, Weinberg DV.** 2002. Accuracy and precision of intraocular injection volume. Am J Ophthalmol **133:**564–566.
30. **Roper SN, Gilmore RL, Houser CR.** 1995. Experimentally induced disorders of neuronal migration produce an increased propensity for electrographic seizures in rats. Epilepsy Res **21:**205–219.
31. **Sato Y, Kobayashi E, Hakamata Y, Kobahashi M, Wainai T, Murayama T, Mishina M, Seo N.** 2004. Chronopharmacological studies of ketamine in normal and NMDA ε1 receptor knockout mice. Br J Anaesth **92:**859–864.
32. **Scallet AC, Schmued LC, Slikker W, Grunberg N, Faustino PJ, Davis H, Lester D, Pine PS, Sistare F, Hanig JP.** 2004. Developmental neurotoxicity of ketamine: morphometric confirmation, exposure parameters, and multiple fluorescent labeling of apoptotic neurons. Toxicol Sci **81:**364–370.
33. **Smiler KL, Stein S, Hrapkiewicz KL, Hiben JR.** 1990. Tissue response to intramuscular and intraperitoneal injections of ketamine and xylazine in rats. Lab Anim Sci **40:**60–64.
34. **Smith W.** 1993. Responses of laboratory animals to some injectable anaesthetics. Lab Anim **27:**30–39.
35. **Society of Veterinary Hospital Pharmacists.** [Internet]. Position statement on compounding of drugs for use in animals. [Cited 30 Mar 2009]. Available at http://www.svhp.org/Compounding-Statement.pdf.
36. **Stabenow JM, Maske ML, Vogler GA.** 2006. Refractometry for quality control of anesthetic drug mixtures. J Am Assoc Lab Anim Sci **45:**60–63.
37. **Steffen BT, Lees SJ, Booth FW.** 2008. AntiTNF treatment reduces rat skeletal muscle wasting in monocrotaline-induced cardiac cachexia. J Appl Physiol **105:**1950–1958.
38. **Thompson JS, Brown SA, Khurdayan V, Zeynalzadedan A, Sullivan PG, Scheff SW.** 2002. Early effects of tribromoethanol,

ketamine/xylazine, pentobarbital, and isoflurane anesthesia on hepatic and lymphoid tissue in ICR mice. Comp Med **52:**63–67.

39. **United States Pharmacopeial Convention.** USP 32. 2008. Baltimore (MD): United Book Press.
40. **Vachon P.** 1999. Self-mutilation in rabbits following intramuscular ketamine–xylazine–acepromazine injections. Can Vet J **40:**581–582.
41. **van Heeckeren AM, Schluchter MD.** 2002. Murine models of chronic *Pseudomonas aeruginosa* lung infection. Lab Anim **36:**291–312.
42. **Vlach KD, Boles JW, Stiles BG.** 2000. Telemetric evaluation of body temperature and physical activity as predictors of mortality in a murine model of staphylococcal enterotoxic shock. Comp Med **50:**160–166.
43. **Welberg LA, Kinkead B, Thrivikraman KV, Huerkamp MJ, Nemeroff CB, Plotsky PM.** 2006. Ketamine–xylazine–acepromazine anesthesia and postoperative recovery in rats. J Am Assoc Lab Anim Sci **45:**13–20.
44. **Wood AK, Klide AM, Pickup S, Kundel HL.** 2001. Prolonged general anesthesia in MR studies of rats. Acad Radiol **8:**1136–1140.
45. **Woodward WR, Choi D, Grose J, Malmin B, Hurst S, Pang J, Weleber RG, Pillers DM.** 2007. Isoflurane is an effective alternative to ketamine–xylazine–acepromazine as an anesthetic agent for the mouse electroretinogram. Doc Ophthalmol **115:**187–201.